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The dynamics of the primordial follicle reserve

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Abstract

The female germline comprises a reserve population of primordial (non-growing) follicles containing diplotene oocytes arrested in the first meiotic prophase. By convention the reserve is established when all individual oocytes are enclosed by granulosa cells. This commonly occurs prior to or around birth, according to species.

Histologically the “reserve” is the number of primordial follicles in the ovary at any given age and is ultimately depleted by degeneration and progression through folliculogenesis until exhausted. How and when the reserve reaches its peak number of follicles is determined by ovarian morphogenesis and germ cell dynamics involving i) oogonial proliferation and entry into meiosis producing an oversupply of oocytes, and ii) large-scale germ cell death resulting in markedly reduced numbers surviving as the primordial follicle reserve. Our understanding of the processes maintaining the reserve come primarily from genetically engineered mouse models, experimental activation or destruction of oocytes, and quantitative histological analysis. As the source of ovulated oocytes in postnatal life, the primordial follicle reserve requires regulation of i) its survival or maintenance, ii) suppression of development (dormancy) and iii) activation for growth and entry into folliculogenesis. The mechanisms influencing these alternate and complex inter-related phenomena remain to be fully elucidated. Drawing upon direct and indirect evidence, we discuss the controversial concept of postnatal oogenesis. This posits a rare population of oogonial stem cells that contribute new oocytes to partially compensate for the age-related decline in the primordial follicle reserve.

Introduction

The concept of a non-renewable primordial follicle pool, assembled around the time of birth in rodents and during gestation in humans, underpins a finite reproductive lifespan and is central to current understanding of ovarian biology. Consideration of the dynamics of the primordial follicle reserve raises more questions than there are answers, however, as although key pathways are emerging, their overall regulation and integration is poorly

understood. The main concepts include i) how the reserve is established, ii) processes causing elimination, iii) regulation of follicle-oocyte dormancy or activation into a growth phase, and iv) possibility of renewal accompanying the age-dependent decline. The significance of the dynamics of the reserve is no more apparent than during ovarian morphogenesis and germ cell development in prenatal life in humans, and perinatally in the mouse and rat. In these growth periods germ cells are produced in large numbers but many are subsequently eliminated, the outcome of which establishes the traditionally-defined primordial reserve. Mechanisms must exist to ensure that the majority of follicles are held intact and remain poised to participate in follicle growth, which in the human is preserved for decades. The reserve faces yet other challenges to its survival from exogenous agents that pose a risk of damage to the oocyte genome with accompanying DNA mutations, or more subtle epigenetic changes. How healthy or faulty oocytes within the reserve are recognized and respectively either preserved or destroyed is a key element impacting the dynamics of the primordial follicles. A reassessment of 'topping up' the reserve by the addition of new primordial follicles from ovarian germline stem cells has emerged in the past ten years. Although this concept has generated a lively debate and a resolution is far from complete, it introduces another factor that potentially affects the dynamics of the reserve. In summary, from the events that shape the establishment of the reserve in prenatal or neonatal ovaries up to the point of its functional exhaustion in adult life, we revisit the concepts of primordial follicle dynamics in the light of recent evidence influencing its stability, depletion or supplementation.

Establishing the primordial follicle reserve

The human fetal ovary

The developing fetal ovary supports the proliferation and maturation of germ cells and their development into primordial follicles. Studies by Block (Block 1951, Block 1952, Block 1953) of fetal, neonatal and adult human ovaries (n=53) using quantitative histological methods provided the first credible estimates of the number of primordial follicles. At 7-9 months gestation (n=10) he reported a range of 350,000 – 1.1 million primordial follicles per pair of ovaries, the average being about 700,000. In postnatal life from 6-9 years (n=5), the average was 500,000 declining to 8,000 between 40-44 years (n=7; (Block 1952)). The age-related fall in follicle supply was not discussed in Block's studies. Within a decade this oversight was corrected when in 1963 a landmark paper by Baker estimated the numbers of all germ cell types (normal and atretic) in human fetal ovaries (n=14). He calculated up to 6.8 million germ cells per pair of ovaries at 5 months gestation declining to about 2 million at the time of birth. The scale of germ cell loss was comparable to the germ cell attrition reported for the rat ovary (Holmes & Mandl 1962, Beaumont & Mandl 1963) suggesting common regulatory mechanisms governing the perinatal supply of primordial follicles. Recent analyses using more accurate stereological methods have expanded on the rate and extent of germ cell proliferation up to 19 weeks gestation (Mamsen *et al.* 2011), reaching nearly 5 million germ cells per ovary at that time, although no distinction is made between stages of development of the germ cells and the extent of inclusion within primordial follicles. Primordial follicles are formed from about 15 weeks gestation in the human fetal ovary (Fig. 1) based on the association of diplotene oocytes with pregranulosa cells (Baker 1963, Forabosco &

Sforza 2007). Their number steadily rises during the second trimester, and plateaus in the third trimester with approximately 350,000-400,000 per ovary at birth. From about 22 weeks, some primordial follicles activate to form the first growing or primary follicles (Maheshwari & Fowler 2008). As far as we know, **the second trimester period of human fetal ovarian development** is the only phase in the history of the dynamics of the reserve where it is increasing in overall number by the addition of oocytes surviving to reach diplotene arrest of meiosis I.

Little is known about the factors responsible for producing this excess of germ cells in the fetal ovary. Array-based studies have described the transcriptome in human fetal ovaries (Fowler *et al.* 2009), potentially allowing identification of regulatory pathways. A network of interacting oocyte transcription factors crucial for oocyte survival and development around the time of follicle formation has been described in the mouse using knock-out models **(Dong *et al.* 1996, Rajkovic *et al.* 2004, Pangas *et al.* 2006)**, with some, such as FIGLA, demonstrated to have comparable expression in the human ovary (Huntriss *et al.* 2002, Bayne *et al.* 2004). Limited functional studies of human fetal ovaries have identified activin A (Martins da Silva *et al.* 2004, Coutts *et al.* 2008, Childs & Anderson 2009) and neurotrophin pathways (Anderson *et al.* 2002, Spears *et al.* 2003, Childs *et al.* 2010a) as likely key determinants of oogonial survival and proliferation and follicle formation (Fig.2). Activin βA is expressed by germ cells in nests and *in vitro* exposure to activin A promotes germ cell survival (Martins da Silva *et al.* 2004). Activin βA expression is lost immediately prior to nest breakdown and follicle formation (Coutts *et al.* 2008), and it is thought that this might act as switch allowing follicle formation

116 involving the de-repression of kit ligand expression (Childs & Anderson 2009). In the
117 mouse, activin A administration *in utero* increased primordial follicle number after birth,
118 although this difference was lost later in life (Bristol-Gould *et al.* 2006a). The BMPs have
119 been suggested to positively regulate oogonial proliferation and survival in the mouse
120 (Pesce *et al.* 2002), but in contrast experimental human data suggests that BMP4
121 increases germ cell apoptosis (Childs *et al.* 2010b), possibly explained by differences in
122 experimental methodology (i.e. isolated germ cells in the mouse vs in their physiological
123 niche in human whole ovary studies).
124 The neurotrophins BDNF and NT4 are expressed by ovarian somatic cells within the cell
125 nests (i.e. presumed precursors to granulosa cells: Fig.2) with both ligands expressed in
126 human but only NT4 in mouse. Mouse knock-out models of the TrkB receptor, targeted
127 by both BDNF and NT4, have resulted in phenotypes including loss of oocytes at the
128 time of follicle formation (Spears *et al.* 2003) and loss of initiation of follicle growth
129 (Paredes *et al.* 2004). Oocyte-derived activin βA regulates BDNF expression in human
130 ovarian somatic cells, and NT4 expression in mouse (Childs *et al.* 2010a), exemplifying a
131 pathway by which the oocyte regulates the surrounding somatic environment, and also
132 demonstrating a conserved pathway between species although involving diverse
133 mediators. Prostaglandin E2 acting on oocytes may also contribute to the regulation of
134 expression of activin βA and BNDF (Bayne *et al.* 2009), and there are undoubtedly other
135 pathways involved. These interactions, derived from experimental human tissue studies,
136 are illustrated in Figure 2.
137 More is known about the circumstances of oocyte death. We use the term ‘circumstances’
138 because of the limited opportunities available for analysis of human material (and no

prospects for *in vivo* experimentation) with most of our knowledge derived from the mouse. Although there are numerous descriptions of specific germ cell types and the timing of their demise in the human fetal ovary that impact on the dynamics of the reserve, the mechanisms responsible remain largely unknown (Maheshwari & Fowler 2008, Hartshorne *et al.* 2009). Much attention has focused on apoptosis (Vaskivuo *et al.* 2001, Fulton *et al.* 2005, Poljicanin *et al.* 2012), although emerging evidence also suggests that the mode of germ cell elimination, especially in meiosis, may be ovary-specific and occurs by several mechanisms not limited to the classic apoptotic pathways (Abir *et al.* 2002). Efforts to identify and quantitate the characteristics of apoptosis as a principal or coherent explanation for oocyte depletion in the human fetal ovary often demonstrate the difficulties and inconsistencies in interpretation of cause and effect, probably due to differential gene expression among cell populations that may be at rest, proliferating, maturing, dying or phagocytosing (Kurilo 1981, De Pol *et al.* 1997, Vaskivuo *et al.* 2001, Abir *et al.* 2002, Hartley *et al.* 2002, Fulton *et al.* 2005, Stoop *et al.* 2005, Albamonte *et al.* 2008, Jaaskelainen *et al.*2010, Boumela *et al.*2011, Poljicanin *et al.* 2012). Nevertheless, these and other studies demonstrate that the Bcl-2 gene family is an important regulator (among other factors) of the balance between survival or death of oocytes prior to primordial follicle formation.

The embryonic and neonatal mouse ovary

Germ cells of the embryonic mouse ovary follow a similar pattern of development as in the human except that it is only after birth that oocytes are fully assembled into the primordial follicle reserve, usually within 2-3 days (Fig 3). In common with the human fetal ovary there is a significant oversupply of oocytes entering meiosis prior to birth,

which is markedly reduced in the perinatal period of development (Fig 4; (Peters *et al.* 1978, McClellan *et al.* 2003, Kerr *et al.* 2006, Pepling 2006, Pepling *et al.*2010). With the advantage of experimental interventions such as the ability to modify gene expression, **much** of our knowledge regarding female germ cell death mechanisms has been generated in the mouse.

Because primordial follicle formation is associated with significant germ cell attrition (Kezele *et al.* 2002, Pepling 2006), investigations into the associated death mechanisms have been topical and numerous laboratories, using both *in vivo* and *in vitro* techniques have concluded that apoptosis (Coucouvanis *et al.* 1993, De Pol *et al.* 1997, Pepling & Spradling 2001, De Felici *et al.* 2008, Xu *et al.*2011) autophagy (Lobascio *et al.* 2007, De Felici *et al.* 2008, Rodrigues *et al.* 2009), and direct extrusion from the ovaries (Rodrigues *et al.* 2009) are all contributory mechanisms of pre- and neonatal oocyte demise. Apoptosis, the most favoured of the three, has been demonstrated not only in mouse models directly targeting Bcl-2 and caspase genes (Bergeron *et al.* 1998, Perez *et al.* 1999, Rucker *et al.* 2000, Flaws *et al.* 2001, Flaws *et al.* 2006, Alton & Taketo 2007, Ghafari *et al.* 2007, Greenfeld *et al.* 2007, Gursoy *et al.* 2008, Ghafari *et al.* 2009) but also because of the findings from several gene knockout (or overexpressor) models belonging to the TNF pathway (Marcinkiewicz *et al.* 2002, Greenfeld *et al.* 2007) , PAR family (Wen *et al.* 2009), and TGFβ family (Kimura *et al.* 2011), all of which actively participate in oocyte loss by regulating apoptosis.

What controls oocyte death to establish the reserve?

For oogonia and oocytes the mechanism of cell death implemented may be related to the signal to die. Most studies of oocyte dynamics in the neonatal mouse ovary point to apoptosis as the mode of death (Ghafari *et al.* 2009, Boumela *et al.* 2011, Hu *et al* 2011.) Therefore, the primordial follicle reserve is presumably established by a balance between the availability of a large number of germ cells and subsequent programmed cell death. Why so many oocytes are produced only to be eliminated remains a mystery, but some possibilities are i) failure of mitosis/meiosis involving **defective** chromosome spindle **functions**, ii) unrepaired DNA damage, iii) insufficient pregranulosa cells, and iv) **degeneration of oocytes during** restructuring of oocyte **cysts** or nests into primordial follicles. The first clues that one member of the p53 gene network had a significant role in controlling oocyte fate came from studies showing that p63, specifically the TAp63α isoform, is expressed uniquely in mouse oocytes and is responsible for their elimination if for example their DNA is damaged (Suh *et al.* 2006). Thus p63 has a role in regulating oocyte survival to establish the primordial follicle reserve. Its expression in late prophase I oocytes but not in early meiotic oocytes or oogonia in fetal ovaries (both mouse and human), suggests a universal role for p63 in protection of the female germline represented by the primordial reserve (Livera *et al.* 2008). In the early postnatal mouse ovary p63 controls oocyte supply by transcriptional induction of BH3-only proteins PUMA or PUMA and NOXA combined (Kerr *et al.* 2012b). These pro-apoptotic Bcl-2 members can initiate oocyte apoptosis either by direct or indirect activation of BAX and BAK. Deletion of *Puma* or *Puma* and *Noxa* together results in an oversupply of primordial follicles in postnatal day 10 mouse ovaries, and deletion of other BH3-only genes, *Bmf* or *Bim* also amplifies the reserve with up to triple the numbers of oocytes

compared with age-matched controls (Fig. 5). The role if any of the other BH3-only proteins remains unknown. Given that 'overstocking' of the primordial reserve in the mouse ovary is wholly or partly the net result of a balance between pro- and anti-apoptotic events, it remains to be shown at what time and which germ cell types (i.e. oogonia and/or oocytes) are affected.

While these studies confirm that apoptotic regulatory mechanisms are key factors in altering the dynamics of the primordial reserve, they do not exclude the possibility of alternate or complementary processes for adjusting the oocyte population. Other studies of the developing human or mouse ovary have demonstrated that the apoptotic paradigm does not satisfactorily account for all aspects of germ cell death (Vaskivuo *et al.* 2001, Abir *et al.* 2002, Alton & Taketo 2007, De Felici *et al.* 2008, Rodrigues *et al.* 2009, Gawriluk *et al.*2011). Alternative modes of cell death that may participate in oogonial-oocyte elimination include autophagy (Guillon-Munos *et al.* 2006, Rubinstein & Kimchi 2012), mitotic arrest (Wartenberg *et al.* 2001) or necroptosis (Vandenabeele *et al.*2010, Christofferson & Yuan 2010).

Dynamics of the postnatal primordial follicle reserve and consequences for reproductive lifespan

Analogous to a stockpile of a precious resource, most oocytes of the primordial reserve are retained as quiescent follicles to support future ovulations throughout the reproductive lifespan. A poorly stocked initial reserve or one in which primordial

follicles are precociously depleted, will result in infertility and in the human, a shortened reproductive lifespan and early menopause (Nelson *et al.* 2013). Current concepts involve progressive loss of human female fertility expressed through subfertility, sterility and the menopause at approximately 10 year intervals (Broekmans *et al.* 2009) . Thus a menopause at age 40 (the traditional definition of the upper limit of 'premature') implies a loss of fertility at 30 and falling fertility from the early 20s. Mathematical analyses of the age-related decline of the non-growing follicle (NGF) reserve (ie. primordial follicles) in human ovaries predicts that if at birth one ovary had 35,000 NGFs, menopause would occur at around 40 years of age but would be delayed to 60 years if the ovary began with 2.5million NGFs (Wallace & Kelsey 2010, Kelsey *et al.* 2012). The number and types of molecules believed to maintain the balance between quiescence and activation of the primordial follicle reserve continue to be discovered chiefly from the study of transgenic mouse models (Reddy *et al.* 2010, Kim 2012, Monget *et al.* 2012, Pangas 2012, Adhikari *et al.* 2013). A key pathway implicated in this is the PI3K pathway, which may have a crucial integrative role linking many of the factors associated with the balance between follicle growth suppression, activation, and the maintenance of healthy quiescence (Fig. 6). Molecules in this pathway include the tuberous sclerosis complex 1 (TSC1) which interacts with phosphatase and tensin homolog deleted on chromosome 10 (PTEN) to maintain quiescence, and the mammalian target of rapamycin (mTORC) which is an activator, and negatively regulated by TSC1 (Zheng *et al.* 2012). Both the oocyte and its pre-granulosa cells are the source and probably the targets for these factors that physiologically exert both stimulatory and inhibitory actions upon the primordial follicle reserve. In addition to intracrine **(factors produced and acting within a cell)** and/or

paracrine inhibition of the recruitment of primordial follicles, an additional 'brake' maintaining their quiescence and perhaps regulating the rate of recruitment may be applied by the growing follicle pool (Barnett *et al.* 2006, Moniruzzaman & Miyano 2010, Reddy *et al.* 2010, Monget *et al.* 2012). Mathematical modeling of histomorphometric data has shown age-dependent differential rates of NGF recruitment in the postnatal human ovary (Wallace & Kelsey 2010) with the great majority of follicles lost in the younger years. Implicit for these observations is the concept that in the early phases of postnatal life including and beyond puberty, some intra-ovarian mechanism limits the decline of the primordial reserve to conserve its stockpile of follicles. In the postnatal mouse ovary it has been suggested that the preservation of a set range of follicle number in the primordial reserve is consistent with a 'quorum-sensing' model (Bristol-Gould *et al.* 2006b, Tingen *et al.* 2009). In this model the ovary can eliminate excess primordial follicles perhaps via a Bcl-2 cell death mechanism but on current evidence cannot add primordial follicles to an otherwise abnormally insufficient reserve. **While biochemical pathways that seem to be involved in the maintenance of primordial follicle health have been proposed based on knock-out models (eg Pdk1 and Rps6: (Reddy *et al.* 2009), how (or indeed whether) primordial follicle health is monitored physiologically is an important but unclear question.**

What is the evidence for a 'brake' applied (at least temporarily) to the disappearance, by growth initiation or direct atresia, of primordial follicles from the reserve? In the Bl/6 mouse strain, following the precipitous decline during the neonatal period, the depletion of primordial follicles per ovary is minimal, losing on average less than 1 follicle per day for up to 14 weeks (Kerr *et al.* 2006, Rodrigues *et al.* 2009) but thereafter declines

277 significantly up to 300 days (Kerr *et al.* 2012a). **Using cell lineage-tracing Lei &**
278 **Spradling {Lei, 2013 #1764} showed that the primordial follicle population is highly**
279 **stable in the postnatal mouse ovary. With an estimated half-life of 10 months in**
280 **adult life, the supply of primordial follicles established in the neonatal ovary is**
281 **sufficient to sustain adult folliculogenesis (and fertility) without a source of renewal**
282 **({Lei, 2013 #1764}).** When growth-initiated i.e. primary follicles are counted, these
283 decline significantly losing about 2.5 follicles on average per day (unpublished data).
284 Could the growing primary follicles and their successors the secondary/antral follicles
285 play a role in restraining recruitment from the primordial reserve? The preferential
286 location of the reserve to the ovarian cortex with growing follicles mostly confined to the
287 medulla (Da Silva-Buttkus *et al.* 2009) suggests a follicle-derived gradient of inhibitory
288 and stimulatory signals that reflects this arrangement. Spatial analysis of primordial
289 follicles has led to the proposal that these follicles inhibit each other by producing as yet
290 unidentified paracrine factors that prevent their activation into primary follicles (Da
291 Silva-Buttkus *et al.* 2009). Perhaps growing follicles influence the rate of entry of
292 primordial follicles into the growth phase, and the phenotype of the AMH knock-out
293 mouse suggests that AMH may contribute to this (Durlinger *et al.* 1999). Analysis of
294 AMH concentrations in relation to NGF number and recruitment across life indicate
295 changing relationships during puberty and early adult life (Fleming *et al.* 2012) in
296 keeping with this factor also playing a significant role in the human. The signal for
297 activation of a reserve follicle may also be based on the origin of the pregranulosa cells
298 and timing of follicle formation, with a separate medullary population formed
299 immediately after birth distinct from the cortical population that supports adult fertility

(Mork *et al.* 2012). This interpretation, based on mouse experimental data, appears to differ from a recent reanalysis of bovine ovarian development (Hummitzsch *et al.* 2013), which indicates that all pregranulosa cells arise early from precursor cells first identifiable within the ovarian surface epithelium.

Thus in the mouse, particularly during the early phase of reproductive life, oocytes destined for ovulation may in theory be supplied mainly from the diminishing primary follicle population. As time passes this temporary stock of growing follicles can by itself no longer sustain the folliculogenic production line and the dwindling size of the early growing follicle population becomes insufficient to exert an inhibitory affect or restraint over the primordial reserve. At that point some of the previously dormant primordial follicles are activated, and the reserve is mobilized. Accessing primordial follicles stored in the reserve will lead ultimately to its depletion whereupon folliculogenesis is curtailed and ovulation ceases. Such detailed information is not available from human studies, which can only be based on cross-sectional analysis of limited data sets. While an increase in the rate of follicle depletion with age is often cited and holds true when expressed as a proportion of remaining follicles, a recent mathematical analysis of the number of follicles leaving the non-growing pool shows that this increases through childhood, peaking at approximately 900 follicles per month at age 14 (with an average follicle endowment), then falling to 600 per month at age 25 and 200 per month at age 35 (Kelsey *et al.* 2012).

The primordial follicle reserve: is it renewable?

In 2004 Johnson et al proposed that in the mouse ovary, the incidence of ongoing, age-related follicle elimination by atresia outstripped the contemporaneous supply available in the primordial follicle reserve. This imbalance was predictive of exhaustion of the reserve within a few weeks beyond puberty (Johnson *et al.* 2004), yet mice may remain fertile for up to 12 months (Gosden *et al.* 1983). To offset the proposed loss of primordial follicles evidence was presented for the existence of ovarian germline stem cells (GSC) capable of proliferation and meiotic maturation into newly-minted oocytes (Johnson *et al.* 2004). Candidate cells were identified in the ovarian surface epithelium leading to the opinion that GSC had been discovered in the mouse (Spradling 2004). Later the notion that GSC arise from the surface epithelium was **revised because the small number (6±3) estimated to be present in the postnatal day 40 ovary was insufficient to generate new oocytes to offset normal follicle loss** (Johnson *et al.* 2005). Other studies of the superficial ovarian cortex reported a mixed population of oocytes, primordial follicles, oogonial-type cells and unidentified cells in mitosis (Kerr *et al.* 2006). In **seeking an alternative source of GSC external to the ovaries**, an origin from bone marrow and blood was next proposed with GSC seeding the mouse ovary to replenish the natural decline in the primordial reserve oocytes (Johnson *et al.* 2005). **This study also reported that in ovaries of mice** exposed to the cytotoxins doxyrubicin (DXR) or histone deacetylase inhibitor trichostatin A (TSA), resulted within 24-36hrs in respective 'spontaneous regeneration' of lost primordial follicles or doubling of their numbers by 'de novo oocyte production'. Together these results were said to reinforce the concept that oogenesis and folliculogenesis could occur in the adult ovary (Johnson *et al.* 2005). **However other studies of the effects of DXR or TSA on mouse ovaries have shown**

depletion of the primordial follicle reserve with no evidence for regeneration (Kujjo *et al.* 2011, Kerr *et al.* 2012a). The contrasting outcomes of gain or loss of primordial follicles reported in different studies adds to the debate on the renewability of germ cells/oocytes in the postnatal ovary, **and it remains the case that even if there is some physiological follicular renewal it too is finite (the incontrovertible existence of the menopause), whether as a result of limiting supply of germ cells, required associated somatic cells or both**. A parabiosis model (Eggan *et al.* 2006) did not provide supportive evidence for a bone marrow or blood-borne source **for ovulated mouse oocytes, but the presence or absence in the ovaries, of marrow- or blood-derived GSC or new follicles was not investigated. When bone marrow obtained from transgenic mice expressing germline-specific green fluorescent protein (GFP) was transplanted into wild-type recipients, GFP-positive germ cells/oocytes were detected in recipient ovaries albeit at a low frequency of 1.4±0.6% of the total immature follicle pool but none developed into ovulated oocytes (Lee *et al*. 2007).** Further studies of the identification and developmental potential of GSC or oogonial stem cells (OSC) in the mouse and human ovary are now available (Zou *et al.* 2009, Pacchiarotti *et al.* 2010, White *et al.* 2012, Zhang *et al.* 2012) but the interpretation of the results continues to generate controversy (Oatley & Hunt 2012, Woods *et al.* 2013). The human data thus far available (White *et al.* 2012) indicate the existence of a small number of cells within the ovary that can be extracted, proliferate *in vitro*, and after labeling and injection into isolated human ovarian cortex tissue, formed primordial follicles containing labeled oocytes. In the mouse, injection of these cells into adult ovary resulted in the ovulation of fertilizable oocytes and livebirths (Zou *et al.*

2009, White *et al.* 2012). This work requires further corroboration, and while potentially of considerable scientific and medical interest, provides no evidence that these cells contribute to physiological ovarian function, including fertility.

If OSC conform to stem cell kinetics they must proliferate by mitosis to preserve their 'stemness'. Genomic analysis in mice of the number of preceding mitotic divisions for antral follicle oocytes revealed how many germ cell divisions have occurred since the zygote stage, this being referred to as oocyte 'depth' (Reizel *et al.* 2012). This study found that oocyte depth increases with age; 13 divisions on average in oocytes sampled at day 30 but 20 divisions in oocytes obtained at 350 days. Do these divisions occur only during embryonic development or throughout all of life?

The first possibility is consistent with the 'production-line' hypothesis (Henderson & Edwards 1968) i.e. the order in which oocytes ovulate postnatally follows the order in which oogonia entered meiosis (and cannot re-enter mitosis) in the embryonic ovary. Meiotic entry is not an 'all-or-none' event but a gradual process occurring from e13.5-e18.5 (Peters *et al.* 1962, Ghafari *et al.* 2007, Ghafari *et al.* 2009) and progressing in the ovary in a cranial-caudal direction (Bullejos & Koopman 2004). Many oogonia in the fetal mouse (and human) ovary continue mitosis whilst others enter meiotic prophase (Evans 1982, Fulton *et al.* 2005) and therefore oogonia with fewer or greater numbers of mitotic divisions would respectively transition early or later into meiosis. Medullary oocytes become early-activated primordial follicles but cortex-resident oocytes are delayed in their assembly as primordial follicles (Fig. 3B). This pattern of germ cell distribution and subsequent dynamics is initiated in the mouse ovary at e13.5 (Byskov *et al.* 1997).

(Woods *et al.* 2012) favour the alternative possibility whereby additional mitoses of OSC during postnatal life produce oocytes of greater 'depth' consistent with measured genetic signatures. Cells with OSC-type properties have been found among primordial follicles in or subjacent to the surface epithelium of the neonatal mouse ovary (Zou *et al.* 2009) and although cells with similar characteristics have been observed (Kerr *et al*. 2006) their identity, function and fate remain to be confirmed. Bristol-Gould et al (Bristol-Gould *et al.* 2006a) and Tingen et al (Tingen *et al.* 2009) reported that 5% of germ cells in the neonatal mouse ovary are 'residual' oogonia, which did not enter meiosis between e13.5-e18.5. If bypassing oocyte nest formation and encapsulation to form primordial follicles, do these orphan oogonia represent the OSC, being rare, unrecognized with routine histology (not being primordial follicles) and problematic to characterize using established stem cell or germline cell markers? Further investigations may reveal if these reputed OSC co-exist with the conventional primordial follicle reserve and represent a hitherto unknown population of germ cells with the potential of development given special opportunity.

Conclusions

From the time of its formation and development within the fetal or neonatal ovary, and throughout the postnatal reproductive lifespan, the primordial follicle reserve is subject to constant change. The remarkable increase then substantial loss of germ cells in the fetal ovary impacts the dynamics of the reserve to the extent of providing oocytes for assembly into primordial follicles. The maximum supply of primordial follicles is the net result of the addition to the reserve of suitably developed oocytes, counterbalanced by depletion

through germ cell death, and depending on species, activation of follicles into a growth phase. Mechanisms controlling germ cell proliferation are not fully understood but evidence is emerging for regulation by interactions between a variety of transcription and growth factors. Elimination of germ cells is likely due to several processes particularly via apoptosis but with increasing evidence for non-apoptotic cell death, such as autophagy, acting alone or in combination with apoptosis and dependant on the type and biological status of the germ cells necessitating their removal. Although in postnatal life many primordial follicles in humans may be preserved for decades in a state of dormancy, the dynamic nature of the primordial follicle reserve is again evident, chiefly through depletion as follicles activate and enter folliculogenesis, and possibly by direct elimination/atresia of those follicles sustaining genomic impairment**. Theoretically, manipulation of the rate of activation of primordial follicle pool could be of clinical value. Temporary increased activation could be of value to women requiring assisted conception later in life to increase the number of oocytes that could be recovered, and conversely slowed activation could be of value to delay the menopause and possibly prolong natural fertility if a reduced pool (and hence increased risk of early menopause) was identified. These possibilities remain remote and, as with all manipulations of the germ line, raise very serious safety considerations.** Recent reports of the existence of a rare population of germline stem cells in mouse and human ovaries have led to suggestions that these cells may partially replenish the reserve as its primordial follicle supply is diminished. If further work confirms recent studies showing that isolated GSC can form follicles with fertilizable

oocytes and viable embryos, this may usher in a new paradigm: an ancillary germ cell population coexisting with the primordial follicle pool, the 'reserve' of the reserve.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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Figure 1. Estimates of germ cell populations of the human fetal ovary based on histomorphometric analysis reported by Baker (1963) and Forabosco & Sforza (2007). Germ cells are always dying, the numbers of atretic germ cells (which include all oogonia and oocytes) being equal to or exceeding the numbers of individual diplotene oocytes or those forming primordial follicles. Primordial follicles begin to form at 15 weeks gestation and at birth the fetal ovary on average contains approximately 400,000 primordial follicles. This represents only 12% of the total germ cell number (healthy and atretic) present at 22 weeks gestation.

Figure 2. Human ovarian development and primordial follicle formation. Illustrative immunohistochemical images to demonstrate (A) proliferative oogonia (mitotic cells

identified by arrows) and oocytes (Oo) within germ cell nests with intermixed NT4-expressing (brown) pregranulosa cells, with the somatic cells (sc) of the stromal regions not expressing NT4 13 weeks gestation. (B) NT4 expression (brown) is also confined to pregranulosa cells within oocyte (Oo) nests and the granulosa cells of newly-formed primordial follicles, with no expression in stromal cells (sc): 21 weeks gestation. (C) Activin βA is expressed by some nests of oocytes (Oo) (green; red nuclear counterstain) but with much weaker expression in others (arrow), indicating synchronous development of oocytes within a nest; 19 weeks gestation. (D) Schematic representation of experimentally-derived interactions between growth factors expressed by oogonia/oocytes of the human fetal ovary and the adjacent pregranulosa cells. Stimulatory (+) and inhibitory (-) regulation as indicated. Scale bar A-C, 20μm.

Figure 3. Development of oocytes and primordial follicles. (A) Pachytene oocytes in e17 mouse ovary showing their arrangement into nests in which individual oocytes are not enclosed by somatic cells that will later become pregranulosa cells of primordial follicles. Scale bar 15μm. (B) Postnatal day 1 mouse ovary showing oocyte nests in the cortex region to the right, and larger individual primordial follicles (pf) in the medulla. Pyknotic structures (example at arrowhead) represent degenerative oocytes. Scale bar 20μm.

Figure 4. Schematic diagram illustrating the general trends of endowment of oocytes and primordial follicles based on stereological analysis in the Bl/6 mouse ovary over indicated ages. Dashed line: is not data based but an estimation of germ cell increase; solid line: mostly based on published data. Oocyte number increases markedly towards

the end of fetal life but many are lost as they assemble to form primordial follicles in the first days after birth. For up to two weeks postnatally primordial follicles decline significantly then enter a period of very slow follicle loss for up to several months followed again by renewed depletion until near or total exhaustion around 12 months of age. Data based in part on Myers *et al.* 2004, Kerr *et al.* 2006, 2012a, Rodrigues *et al* . 2009, Lei & Spradling 2013.

Figure 5. Gene regulation of the primordial follicle reserve in the mouse ovary. Comparison of primordial follicle supply in postnatal day 10 mouse ovaries in wild-type, *p53* -/- and various BH3 member knockout models. Based on data from Kerr *et al* 2012b.

Figure 6. Schematic diagram illustrating the options available for primordial follicles leaving the arrested reserve (growth/suppression of growth; maintenance of health; elimination and possibly renewal) and representative proteins or genes identified as regulators of these various pathways.

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Figure 1

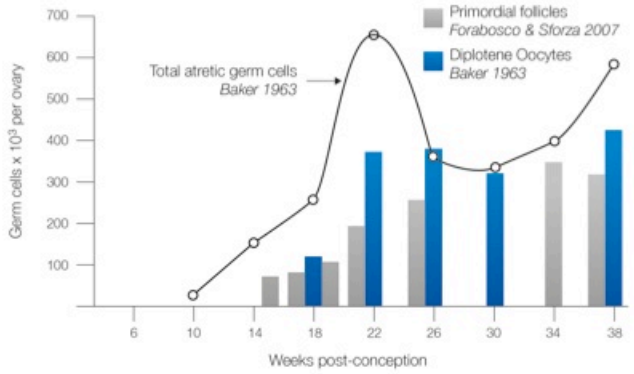
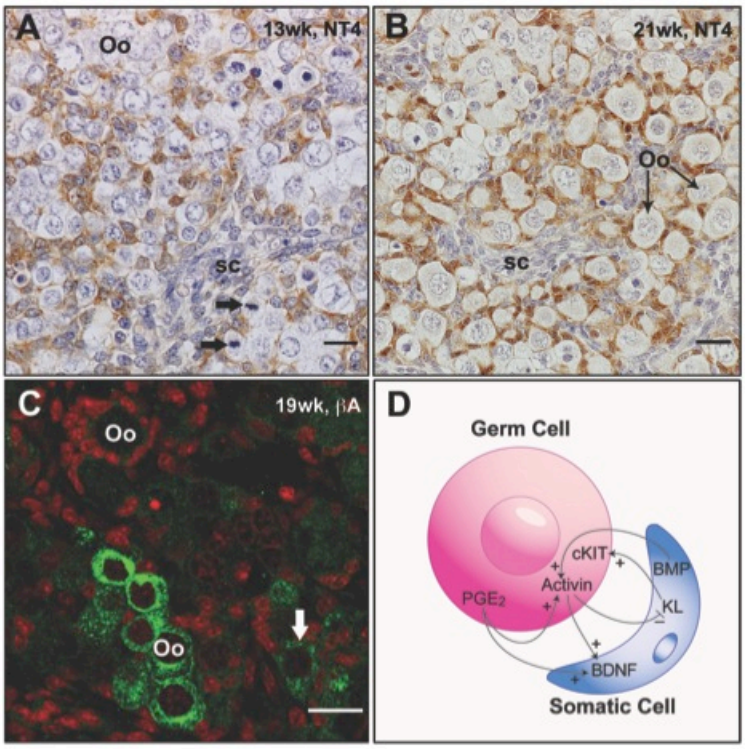


Figure 2



819

Figure 3

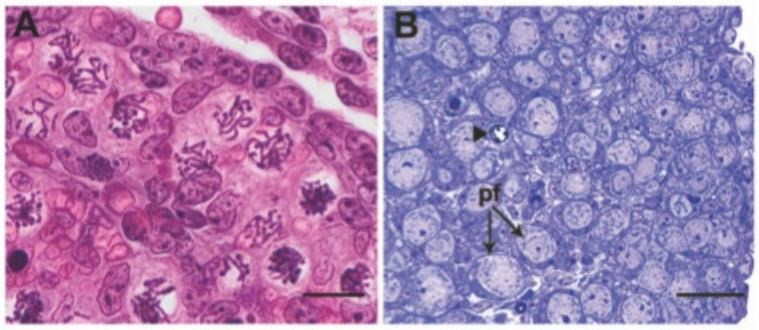
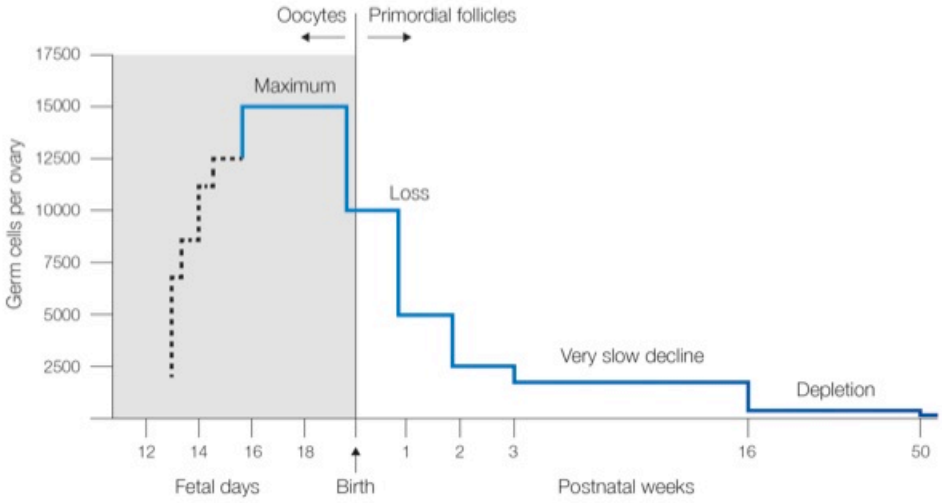


Figure 4



820

Figure 5

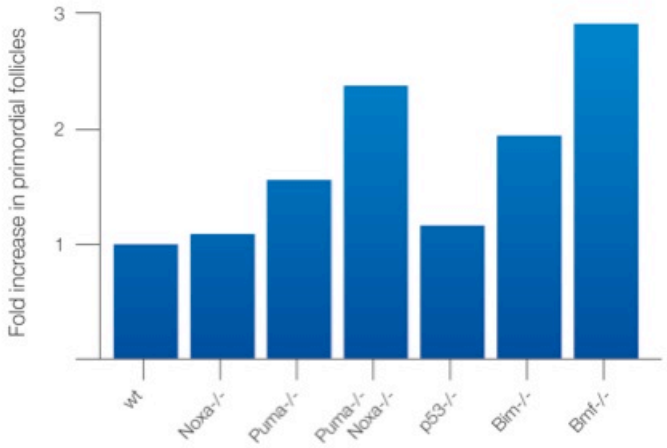
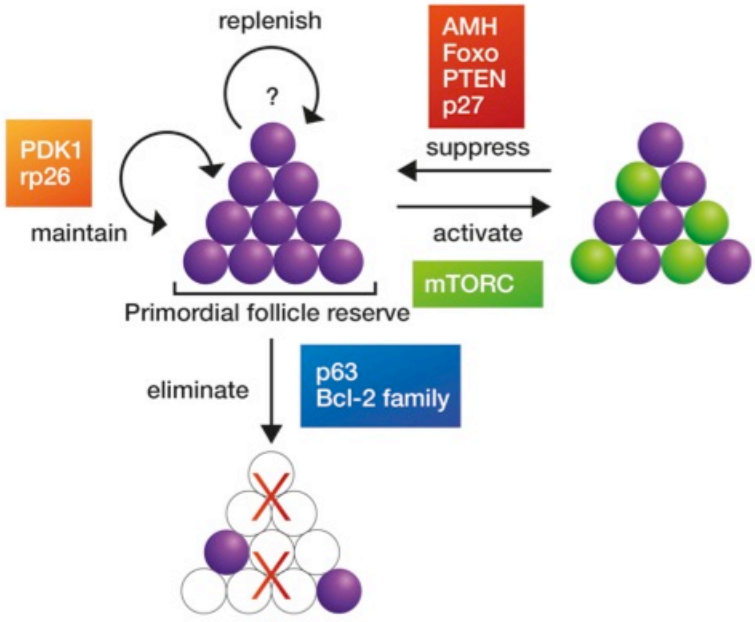


Figure 6



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